Pharm Res (2014) 31:2178–2185
DOI 10.1007/s11095-014-1317-3

RESEARCH PAPER

Liposomal Co-Delivery of Omacetaxine Mepesuccinate and Doxorubicin for Synergistic Potentiation of Antitumor Activity

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Received: 5 August 2013 / Accepted: 28 January 2014 / Published online: 22 February 2014

ABSTRACT

Purpose Anticancer chemotherapy usually involves the administration of several anticancer drugs that differ in their action mechanisms. Here, we aimed to test whether the combination of omacetaxine mepesuccinate (OMT) and doxorubicin (DOX) could show synergism, and whether the liposomal co-delivery of these two drugs could enhance their antitumor effects in cervical carcinoma model.

Method OMT-loaded liposomes (OL) were prepared by loading the drug in the lipid bilayers. OL were then electrostatically complexed with DOX, yielding double-loaded liposomes (DOL). DOX-loaded liposomes (DL) were formulated by electrostatic interaction with negatively charged empty liposomes (EL). The combination index (CI) values were calculated to evaluate the synergism of two drugs. *In vitro* antitumor effects against HeLa cells were measured using CCK-8, calcein staining, and crystal violet staining. *In vivo* antitumor effects of various liposomes were tested using HeLa cell-bearing mice.

Results Combination of DOX and OMT had ratio-dependent synergistic activities, with very strong synergism observed at a molar ratio of 4:1 (DOX:OMT). The sizes of EL, DL, OL, and DOL did not significantly differ, but the zeta potentials of DL and DOL were slightly higher than those of OL and EL. *In vitro*, DOL showed higher antitumor activity than OL, DL or EL in cervical carcinoma HeLa cells. *In vivo*, unlike other liposomes, DOL reduced the tumor growths by 98.6% and 97.3% relative to the untreated control on day 15 and 25 after the cessation of treatment, respectively.

Conclusions These results suggest that liposomal co-delivery of DOX and OMT could synergistically potentiate antitumor effects.

KEY WORDS antitumor effects · co-delivery · combination therapy · liposomes · omacetaxine mepesuccinate

ABBREVIATIONS

CI	combination index
DL	doxorubicin-loaded liposomes
DOL	doxorubicin and omacetaxine mepesuccinate-loaded liposomes
DOX	doxorubicin
EL	empty liposomes
OL	omacetaxine mepesuccinate-loaded liposomes
OMT	omacetaxine mepesuccinate

INTRODUCTION

Co-treatments with two or more chemical anticancer agents that differ in their pharmacological action mechanisms are often used in clinical practice for anticancer therapy (1–3). The anthracyclin anticancer agent, doxorubicin (DOX), has been used in combination with other chemotherapeutics, such as bleomycin, and cyclophosphamide (4,5). Omacetaxine mepesuccinate (OMT) is a first-in-class cephalotaxus alkaloid drug that was approved in 2012 by the Food and Drug Administration (FDA) for the treatment of chronic or accelerated-phase chronic myeloid leukemia (6). OMT inhibits protein translation by interrupting the initial translation step (7,8), and also decreases the expression levels of short-half-life proteins, such as the antiapoptotic protein, Mcl-1 (9).

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Due to its novelty, few studies have examined the use of OMT with other anticancer drugs for the potentiation of their anticancer effects. Moreover, most OMT studies have focused on the anticancer effects against chronic myeloid leukemia (7,10), and little studies have been done on the effects of OMT to other types of solid tumors such as cervical carcinoma.

A recent study showed that anticancer effects were enhanced *in vitro* by co-treatment of Mcl-1-specific short interfering RNA (siRNA) plus doxorubicin (DOX) to human breast cancer cells (11). Another study reported that the *in vitro* co-treatment of an Mcl-1-downregulating chemical plus DOX sensitized chronic lymphocytic leukemia cells to the anticancer effects of these drugs (12). Given the ability of OMT to decrease Mcl-1 levels (9,13), these studies prompted us to speculate that the co-administration of OMT plus DOX could enhance the tumor cell-killing effects of these agents.

In the co-administration of anticancer agents, the simultaneous delivery of both chemotherapeutics to the tumor tissues at the most effective ratio should enhance the therapeutic outcome. Moreover, the synergistic co-delivery of chemotherapeutics to the target tissues can reduce the administration doses as well as side effects (14). To enable such co-delivery, various nanoparticulate drug delivery systems have been employed. For example, the polymeric micelle-based co-deliveries of disulfiram with DOX derivatives have been studied as anticancer combination therapies (15). Double-walled microspheres consisting of poly (lactic-co-glycolic acid) and poly (lactic acid) were designed to co-deliver DOX along with a p53-encodding gene (16). Lipid-based nanocarriers were designed to co-deliver DOX and Bcl-2-specific siRNA (17). Cationic liposomes have been used to co-deliver Mcl-1-specific siRNA and suberoylanilide hydroxamic acid to tumor tissues (18).

Among various nanocarriers for co-delivery (14,19), liposomes may have several advantages over other carriers. The lipid bilayer structure of liposomes with aqueous inner phase allows the co-delivery of hydrophobic drug in the lipid bilayers with hydrophilic drug in aqueous phase. Moreover, the easily modulated surface charges of liposomes make it possible for co-delivery of hydrophobic or hydrophilic drugs inside liposomes with charged drug bound to the liposomal surfaces by electrostatic interaction (20). The versatile capacity of liposomes for carrying various drugs with different physicochemical properties may facilitate the use of liposomes for combination therapy. Especially, DOX with positive charge at neutral pH can be loaded to the negatively charged liposome surfaces, and OMT with lipophilic property can be loaded into lipid bilayers during liposome preparation procedure. Moreover, unlike some polymeric nanocarriers where drugs are chemically conjugated for co-delivery (15,21), liposomal loading of drugs without chemical modification eliminates the regulatory concerns on the altered therapeutic efficacy and safety issues of modified anticancer drugs.

In this study, we thus tested whether OMT, which decreases Mcl-1 levels, may potentiate the anticancer effects of DOX and if so whether liposome nanocarrier-based co-delivery could provide the synergistic effects of OMT and DOX *in vitro*, and *in vivo* cervical carcinoma model.

MATERIALS AND METHODS

Cell Culture

The HeLa (human cervical carcinoma) cell line (American Type Culture Collection, Rockville, MD, USA) was cultured in Dulbecco's Modified Eagle's Medium (Gibco BRL Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Evaluation of Synergism Between OMT and DOX at Various Ratios

To test the synergistic anticancer activity of OMT in combination with DOX *in vitro*, HeLa cells (4×10^4 cells/well) were seeded onto 48-well plates, cultured overnight, and then treated with various concentrations of DOX (0.1 to 100 μM) or OMT (0.1 to 100 μM). For the combination index assay, wells were co-treated with two drugs at molar ratios of 1:1, 2:1, 4:1 or 8:1 (DOX:OMT) by fixing the concentration of OMT as 0.25 μM. After 24 h, cell viability was assessed by CCK-8 assays (Dojindo, Tokyo, Japan). Briefly, 20 μl of CCK-8 (water-soluble tetrazolium salt) solution was added to each well for 30 min, and absorbance was measured at 450 nm using a microplate reader (Sunrise-Basic TECAN, Männedorf, Switzerland). The cell viability in each group was expressed as a percentage of that in control cells. The synergism of DOX and OMT co-treatment was evaluated by calculating the combination index (CI) using a Calcusyn software (Biosoft, Cambridge, UK) (22,23), with a $CI<1.0$ considered to represent synergism, and with a CI between 0.1 and 0.3 to indicate strong synergism. A $CI<0.1$ represents very strong synergism (24,25).

Preparation of Liposomes

Liposomes were prepared using a slight modification of the previously described method for preparing multi-lamellar vesicles (26). To prepare empty liposomes (EL), egg L-α-phosphatidylcholine (PC; Avanti Lipids, Birmingham, AL, USA), egg L-α-phosphatidyl-DL-glycerol (PG; Avanti Lipids), and cholesterol (Chol; Avanti Lipids) were mixed at a molar ratio of 7:3:5 with total 15 μmole of lipids. To prepare OMT-loaded liposomes (OL), 50 mM of OMT in methanol was combined with the mixed lipids at a molar ratio of 7:3:5:0.16

(PG:PC:Chol:OMT). A rotary evaporator was used to remove the organic solvents, and the resulting thin films were hydrated by being vortexed with 3 mL of HEPES-buffered saline (HBS, pH 7.4). The resulting multi-lamellar vesicles were extruded three times through 0.1-μm polycarbonate membrane filters (Millipore, Bedford, MA, USA).

To load DOX onto the liposomes by electrostatic interaction, 130 μg of DOX (Sigma, St. Louis, MO, USA) was added to 1 mL of EL or OL with 5 mM phospholipids. Unloaded DOX was removed by gel filtration through a Sephadex G-25 M column (GE Healthcare, Piscataway, NJ, USA), yielding DOX-loaded liposomes (DL) or DOX-loaded OL (DOL). The loading efficiency of OMT in OL or DOL was measured by UV spectroscopy at 280 nm. The loading efficiency of DOX in DL or DOL was spectrophotometrically determined at 490 nm.

Measurement of Particle Sizes and Zeta Potentials

The particle sizes of the generated liposomes were measured using dynamic light scattering. The liposomes were diluted with HBS (pH 7.4) and placed in an ELS-Z (Photal, Osaka, Japan). The hydrodynamic diameters of the particles were determined by He-Ne laser (10 mW) light scattering, and the zeta potentials were determined by laser Doppler micro-electrophoresis at an angle of 22°. Data were analyzed using the ELS-Z software (Photal).

Testing the *In Vitro* Antitumor Activities of Liposome Formulations

CCK-8 assays, calcein staining, crystal violet staining, and were used to evaluate the *in vitro* antitumor activities of the various liposomes. Annexin V staining was used to test the apoptosis-mediated death of cancer cells (27). HeLa cells (4×10^4 cells/well) were seeded onto 48-well plates, incubated overnight, and then treated for 24 h with EL, DL, OL, or DOL at concentrations of 1 μM for DOX and 250 nM for OMT (i.e., 4:1). For CCK-8 assay, 20 μl of CCK-8 (water-soluble tetrazolium salt) solution was added to each well for 30 min, and absorbance was measured at 450 nm using a microplate reader (Sunrise-Basic TECAN, Männedorf, Switzerland). The cell viability in each group was expressed as a percentage of that in control cells. For calcein staining, the culture medium was removed, the cells were washed twice with phosphate-buffered saline (PBS), and 200 μl of calcein solution (2 mM) was added to each well. After 30 min, the cells were washed twice with PBS and observed under a fluorescence microscope (Leica DM IL; Leica, Wetzla, Germany). For crystal violet staining, the liposome-treated cells were washed twice with PBS, added with 200 μl of staining solution (0.5% crystal violet and 20% methanol), and photographed with a digital camera (Canon PC1089; Canon, Tokyo, Japan). For Annexin V staining, the cells were resuspended in Annexin V binding buffer (BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I, BD Bioscience, San Jose, CA, USA), and treated with 5 μl of fluorescein isothiocyanate-Annexin V solution for 20 min. The cells were then analyzed by a BD FACS Calibur using Cell Quest Pro software (BD Bioscience).

Testing the *In Vivo* Antitumor Effects of the Liposome Formulations

The *in vivo* antitumor effects of various liposomes (EL, OL, DL, and DOL) were tested in HeLa-tumor-bearing nude mice. All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Seoul National University (Seoul, South Korea; approved animal experimental protocol number SNU-130129-3-1). Six-week-old female athymic nude mice (Orient Bio, Kyonggi-do, South Korea) were subcutaneously injected with 3×10^6 HeLa cells at the dorsal aspect of the left side of each mouse. When the tumor volume reached 150–200 mm^3, the mice were intratumorally administered every 3 days with EL, 0.24 mg/kg of OMT in free form or liposomes (OL, DOL), and 1.0 mg/kg of DOX in free form or liposomes (DL, DOL). For each group, five mice were used. All mice received a total of four doses, given on days 11, 14, 17 and 20. Tumor size were measured in two dimensions using a slide caliper, and the tumor volume as a % b % 0.5, where a is the largest and b is the smallest diameters.

Statistics

Statistical analyses were performed using the Student *t*-test or ANOVA, with the Student–Newman–Keuls test employed as a post hoc test. The SigmaStat software, version 3.5 (Systat Software, Richmond, CA, USA) was used for all analyses, and $P<0.05$ was considered significant.

RESULTS

Synergistic Effects of DOX Plus OMT Co-Treatment *In Vitro*

Co-treatment of HeLa cells with DOX and OMT showed ratio-dependent synergism. Treatment of cells with DOX (Fig. 1a) or OMT (Fig. 1b) alone concentration-dependently increased HeLa cell death. The concentrations capable of inhibiting HeLa cell survival by 50% (IC50) were 3.2 μM for DOX and 0.14 μM for OMT. Co-treatment of HeLa cells with various DOX:OMT ratios followed by calculation of the CI revealed that the cell-killing effects were ratio-dependent (Fig. 2). At all tested combination ratios of 1:1, 2:1, 4:1, and 8:1, the CI values were <1.0, indicating the presence of

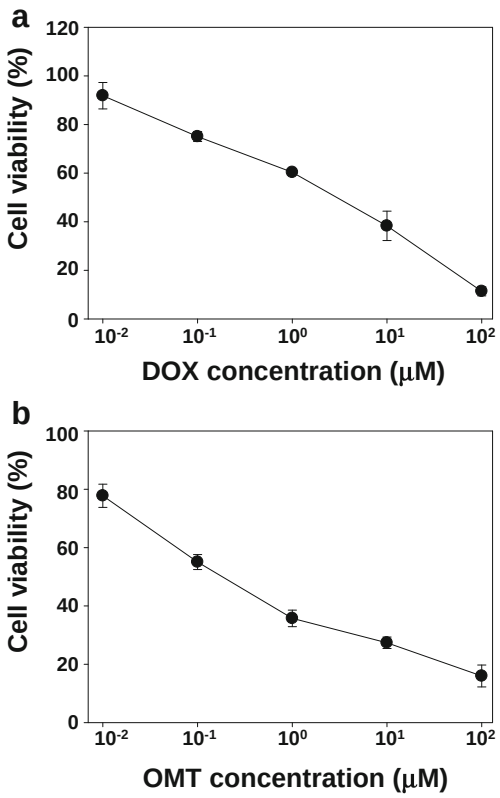

Fig. 1 *In vitro* antitumor effects of free DOX and OMT. HeLa cells were treated with (**a**) free DOX or (**b**) OMT at various concentrations. After 24 h, cell viability was measured using CCK-8 assays. The results are the mean ± SE of four independent experiments.

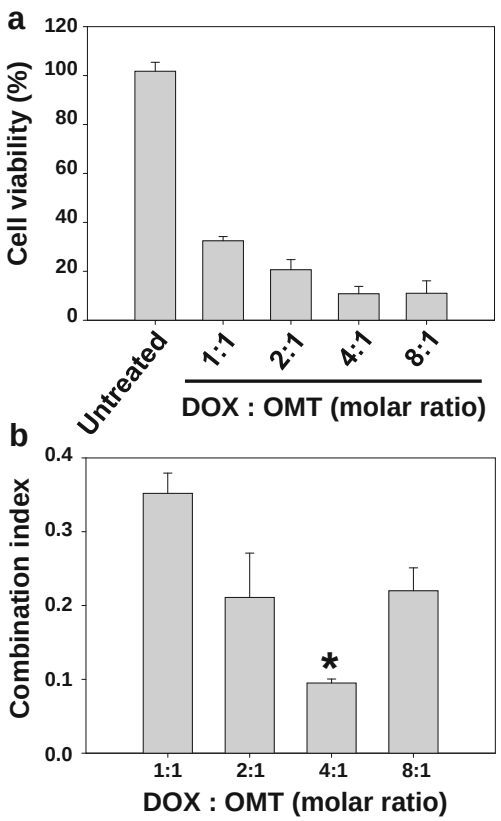

Fig. 2 CI values for free DOX and OMT. HeLa cells were co-treated with DOX and OMT at various molar ratios. (**a**) After 24 h, cell viability was measured by CCK-8 assay. (**b**) CI values were calculated based on the dose–response curves of DOX and OMT at various molar ratios. The results are the mean ± SE of four independent experiments.

synergism between the two drugs. Among the groups, the highest CI value (0.35±0.03) was observed for DOX:OMT combination ratio of 1:1. DOX:OMT ratios of 2:1 and 8:1 yielded CI values in the range of 0.1 to 0.3, indicating strong synergism. Meanwhile the lowest CI value (and thus the strongest synergism) was associated with the DOX:OMT ratio of 4:1 (CI=0.09±0.01), representing 'very strong' synergism.

Physicochemical Characterization of Drug-Loaded Liposomes

Based on the CI values of DOX and OMT, DOL was formulated to have the molar ratios of DOX and OMT as 4:1. For comparison with DOL, EL, DL, and OL were prepared. DL was formulated by loading DOX onto the surface of negatively charged EL (Fig. 3a). OL was prepared by entrapping OMT into the lipid bilayers of EL (Fig. 3b). DOL was made by loading DOX onto negatively charged OL, at a 4:1 molar ratio of DOX:OMT (Fig. 3c). There was no significant size difference among the various liposomes (Fig. 3d). The zeta potentials of DL and DOL were higher than those of EL and OL, while the potentials of EL and OL did not significantly differ (Fig. 3e). Table I summarizes the physicochemical properties of four types of liposomes used in this study.

In Vitro Antitumor Effects of Liposome-Mediated Co-Delivery of DOX and OMT

DOL exerted higher antitumor effect as compared to other groups. The *in vitro* antitumor effects of liposomal co-delivery of DOX/OMT were evaluated by CCK-8 cell viability assays (Fig. 4a), fluorescent live-cell staining (Fig. 4b-e), and crystal violet staining (Fig. 4f). Our results revealed that HeLa cell viability was significantly reduced by treatment with DL, OL, or DOL compared to EL (Fig. 4a). CCK-8 assay revealed the lowest cell viability in DOL-treated cells, with 10.9±2.2% viable cells. Consistent with this finding, the DOL-treated group showed the lowest proportion of live cells, as assessed by calcein-based fluorescence staining (Fig. 4e), and crystal violet staining (Fig. 4f). The apoptosis of liposome-treated cells was assessed by Annexin V-staining (Fig. 4g). As compared to the DL-treated cells, OL-treated cells showed 2.7-fold higher apoptotic population of cells, with 73.8±0.7% of Annexin V-positive cells. The highest population of apoptotic cells was observed in DOL-treated group showing 88.9±0.7% Annexin V-stained cells.

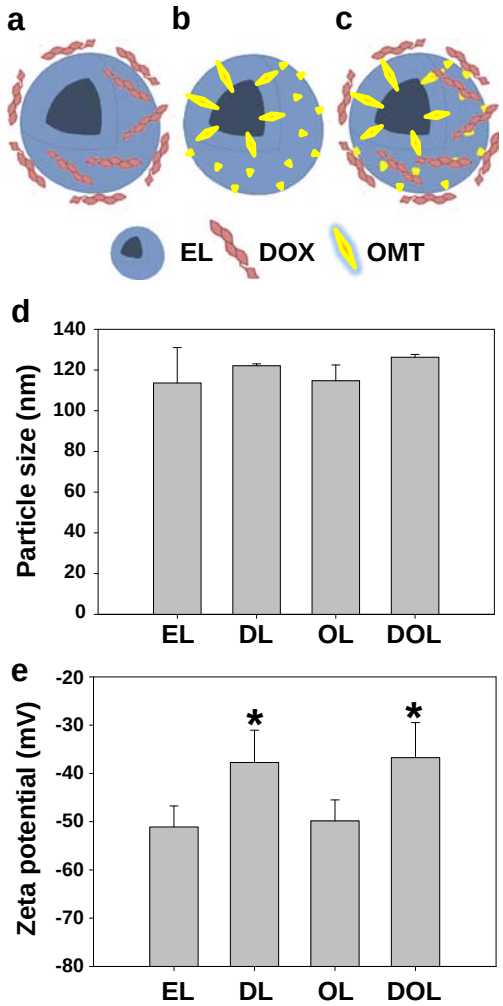

Fig. 3 Schematic illustrations of liposomes and characterization of DOL. Schematic illustrations of (**a**) DL, (**b**) OL, and (**c**) DOL. (**d**) The particle sizes of the various liposomes were measured by dynamic light scattering. (**e**) The zeta potentials of various liposomes were determined using an electro-Doppler method. *: significantly higher than the EL or OL groups ($P < 0.05$). The results are the mean ± SE of four independent experiments

In Vivo Antitumor Effects of Liposomal Co-Treatment with DOX and OMT

Co-delivery of DOX and OMT via DOL showed the enhanced and prolonged *in vivo* antitumor effects as compared to other groups. Fifteen days after completion of four times of repeated intratumoral administration of liposomes to HeLa-tumor-bearing mice (Fig. 5a), tumor volume of EL-treated group did not differ from that of untreated mice, whereas the groups treated with OL and DL showed 63.1% and 51.8% inhibition of tumor growth compared to untreated mice, respectively (Fig. 5b). However, this inhibitory effect of OL and DL was temporary, as the tumor sizes on day 25 after completion of liposome administration (i.e. day 45 after tumor cell inoculation) did not differ in the OL, DL, and untreated groups. In contrast, DOL-treated mice showed significant and prolonged antitumor effects (Fig. 5c), exhibiting a 98.5% and 97.3% reduction in average tumor volume compared to untreated mice on day 35 and 45 after inoculation of tumor cells, respectively.

DISCUSSION

In this study, we demonstrate that DOX/OMT co-treatment has very strong synergistic effects against cancer cells, and further provide evidence that the liposomal co-delivery of DOX and OMT can potentiate their antitumor effects *in vitro* and *in vivo*.

We observed that OMT and DOX exerted synergistic antitumor effect against cervical carcinoma HeLa cells. OMT has been known as an inhibitor of protein translation to interrupt initial translation step by interaction with A-site of ribosome (7). In addition, OMT is known to decrease the expression levels of anti-apototic Mcl-1, leading to the cancer cell death (13). The reduction of Mcl-1 by siRNA specific for Mcl-1 has been reported to exert anticancer activity (28). Moreover, several studies demonstrated that over-expression of Mcl-1 contributes drug resistance of cancer cells (29,30) and that the reduction of Mcl-1 expression could sensitize the cancer cell-killing effects of various chemotherapeutics including 5-fluorouracil (31), cisplatin (31), mitoxantrone (29), and DOX (11,32). In particular, it has been reported that co-treatment of OMT and DOX induced remarkable anticancer effect showing extended survival as compared to the single treatment in Eμ-Myc lymphoma tumor model (32). The study suggested that cancer cells could be sensitized to DOX when the expression levels of Mcl-1 conferring drug resistance were reduced due to the treatment of OMT. Recent study also

Table I Physicochemical Properties of Various Liposomes

	Particle size (nm)	Zeta potential (mV)	Loading efficiency (%)		Drug content (μg/ml)	
			DOX	OMT	DOX	OMT
EL	113.6 ± 17.4	−51.1 ± 4.3	–	–	–	–
DL	122.1 ± 1.0	−37.7 ± 6.7	90.1 ± 2.3	–	117.1 ± 3.0	–
OL	114.7 ± 7.7	−49.8 ± 4.3	–	93.2 ± 2.7	–	27.0 ± 0.8
DOL	126.3 ± 1.4	−36.7 ± 7.3	89.8 ± 3.5	92.9 ± 1.9	116.7 ± 4.6	26.9 ± 0.6

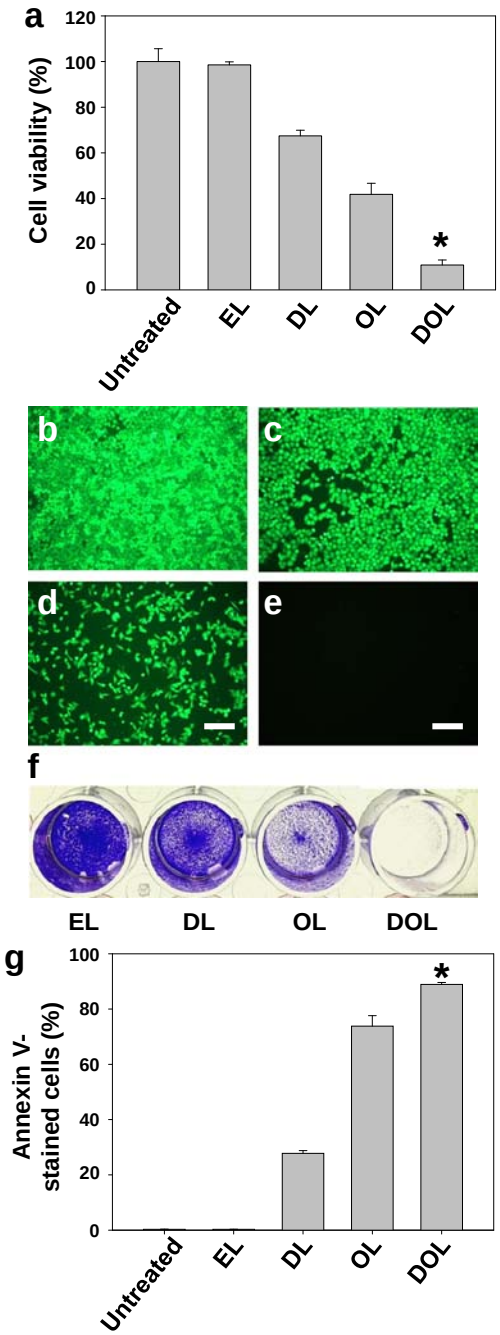

Fig. 4 *In vitro* tumor cell-killing effects of DOL. HeLa cells were treated with EL, DL, OL or DOL (molar ratio 4:1, DOX:OMT). (**a**) After 24 h, viable cells were measured using CCK-8 assays, calcein staining (**b**, EL; **c**, DL; **d**, OL; **e**, DOL), or (**f**) crystal violet staining. (**g**) Annexin V-stained cell populations were analyzed by flow cytometry. The results are the mean ± SE of four independent experiments. Bar size: 100 μm. *: significantly lower than the other groups ($P < 0.05$).

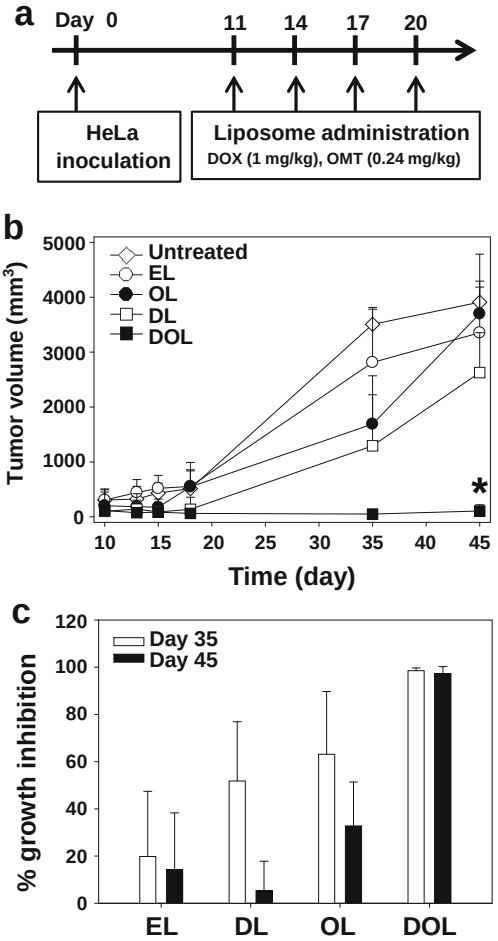

Fig. 5 *In vivo* antitumor effects of DOL. (**a**) HeLa cell-xenografted mice were injected intratumorally with EL, OL, DL or DOL every 3 days on four occasions beginning on day 11 after tumor cell inoculation. (**b**) Tumor volumes were periodically measured using calipers. For each group, five mice were used. The results are expressed as the mean ± SD. *: significantly lower than other groups at day 45 ($P < 0.05$). (**c**) Percentage tumor growth inhibition after treatment ($n = 5$). The results are expressed as the mean ± SD.

demonstrated that expression levels of Mcl-1 increased after prolonged exposure of drug-resistant breast cancer cells to DOX, leading to resistance, and siRNA-mediated silencing of Mcl-1 enhanced the cancer cell-killing effects of DOX in these resistant cells (11). Based on the previous studies, the synergistic anticancer effect of DOL could be due to the enhancement of DOX activity by reduction of Mcl-1 by OMT.

At a molar ratio of 4:1 (DOX:OMT), the CI was 0.09± 0.01, indicating very strong synergism in Hela cells and suggesting that liposomal co-delivery of DOX and OMT could be useful as a chemotherapeutic strategy. A recent study reported that co-treatment of human breast cancer (MCF-7) cells with DOX and the anti-malarial drug, dihydroartemisinin (1:2 molar ratio) yielded CI of 0.50±0.05 (33). The combination of DOX and the cyclin-dependent kinase inhibitor, P276-00, was tested at a 1:12 ratio against various non-small cell lung carcinoma cell lines (34). In the study, the CI ranged from 0.63 to 0.94 depending on the types of non-small cell lung cancer cells.

We also addressed a potential delivery strategy for our co-treatment. In 2012, OMT was finally approved in the USA, but only for subcutaneous injection (6,35). In clinical trials, short intravenous infusions of OMT were associated with intense cardiovascular complications, hypotension and tachycardia. Although dose-limiting toxicities are regarded as a major obstacle in the clinical use of chemotherapeutic anticancer drugs, this hurdle can often be overcome by the use of liposomal formulations (36,37). For example, Doxil is the first liposomal anticancer agent to be approved for the treatment of solid tumors. By liposomal formulation of DOX in Doxil, the cardiotoxicity could be avoided (36). In addition, the liposomal co-delivery of synergistic two drugs may provide simultaneous delivery to the same cells in tumor tissues, enhancing the therapeutic effects.

Here, we assessed the liposomal delivery of our co-treatment by first encapsulating OMT into a lipid bilayer, and then loading DOX onto the negatively charged liposome surface via electrostatic interactions. Electrostatic interaction-based loading of liposomes has been mainly utilized for the delivery of nucleic acids, such as siRNA (26) and plasmid DNA (38). The use of electrostatic interaction simplifies the loading process down to a single step and increases the amount that can be loaded, making it highly suitable for 'bench-to-market' development (18).

As lipid components of DOL, we used PG, PC, and Chol, which are biodegradable and biocompatible. PC and Chol have been used as main lipid components of commercialized DOX liposomes (Myocet™) approved for treatment of metastatic breast cancer (39). PG has been used as a lipid component of verteporfin liposomes (Visudyne™) approved for wet age-related macular degeneration (39). From the regulatory perspective, current clinical use of PG, PC, Chol as components of approved liposome products suggests that DOL may be further developed for clinical use with relatively lower safety concerns as compared to other novel material-based nanocarriers lacking the history of clinical use.

In vivo, we observed near-complete and prolonged regression of tumor growth in DOL-treated mice (Fig. 5b, c). This strong antitumor effect suggests that our liposomal formulation can deliver OMT and DOX at the optimal ratio, maximizing the effects of this synergistic combination. Although OMT single treatment was approved by FDA for treatment of leukemia, our results done for *in vivo* cervical carcinoma HeLa xenograft mouse model indicates the feasibility that OMT can be used to treat other types of cancers upon combination with conventional chemotherapeutics. Moreover, our results obtained from intratumoral injection suggest that future studies are warranted to study the safety of liposome *versus* free OMT for systemic administration.

In conclusion, we herein report that DOX and OMT show very strong synergism in cervical carcinoma model. *In vitro*, DOL formulated at the optimal ratio showed much higher antitumor activity against HeLa cells compared to DL and OL. In HeLa tumor-bearing mice, liposomal co-delivery of DOX and OMT provided near-complete and prolonged antitumor activity over 25 days after the cessation of treatment. These findings suggest that the liposomal co-delivery of DOX and OMT may potentiate their anticancer effects in cervical cancer model, and may be appropriate for clinical use in the future.

ACKNOWLEDGMENTS AND DISCLOSURES

Gayong Shim and Sangbin Lee contributed equally to this work. This work was supported by research grants from the Ministry of Science, ICT and Future Planning (No. 2013035166; 2013036131), from the Korean Health Technology R&D project, Ministry of Health and Welfare (No. A092010), and from Business for Cooperative R&D between Industry, Academy, and Research Institute funded Korea Small and Medium Business Administration (No. C0010962).

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